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TITLE: Identifying Physiological Substrates of the Snf-1-Related Kinase Hunk: New Biomarkers of Kinase Activity and Therapeutic Targets in Metastatic Breast Cancer

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14. ABSTRACT
Kinases play key roles in the etiology of most cancers. Knowing what proteins are phosphorylated by these kinases is necessary to understand how aberrant kinase activity is linked to disease, to reveal new therapeutic targets, and to anticipate effects of kinase inhibition. There is currently an intense interest in identifying therapeutic strategies to regulate kinase activity in cancers, typically using small molecule inhibitors. Highly successful small molecule kinase inhibitors are already in use to treat several forms of cancer. It is relatively straightforward to identify lead small molecule kinase inhibitors, however cost issues preclude the majority from ever reaching patients. To expedite the flow of targeted anticancer agents to patients, the FDA has approved using Phase 0 trials, in which a primary endpoint is to evaluate target modulation in a small number of patient samples to establish mechanism of action. It is absolutely critical to identify direct physiological substrates of kinases so that anti-phosphosite antibodies can be derived to determine if exploratory kinase inhibitor drugs are truly hitting their intended targets in patients. A recent study demonstrated convincingly that the Snf-1-related serine/threonine kinase Hunk is essential for autochthonous tumor metastasis in a MYC-driven breast cancer model. Another recent study reported the development of a novel, efficient approach to identify true physiological kinase substrates from any tissue or cell source termed the Reverse In-gel Kinase Assay (RIKA). The goal of the work in this project was to bring these two advancements together to identify Hunk substrates, providing the first look downstream of the only kinase known to be required for breast cancer metastasis and to inform strategies to block Hunk activity in breast cancer.

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INTRODUCTION

Kinases play key roles in the etiology of most cancers. Knowing what proteins are phosphorylated by these kinases is necessary to understand *how* aberrant kinase activity is linked to disease, to reveal new therapeutic targets, and to anticipate effects of kinase inhibition. There is currently an intense interest in identifying therapeutic strategies to regulate kinase activity in cancers, typically using small molecule inhibitors. Highly successful small molecule kinase inhibitors are already in use to treat several forms of cancer. It is relatively straightforward to identify lead small molecule kinase inhibitors, however cost issues preclude the majority from ever reaching patients. To expedite the flow of targeted anticancer agents to patients, the FDA has approved using Phase 0 trials, in which a primary endpoint is to evaluate target modulation in a small number of patient samples to establish mechanism of action. It is absolutely critical to identify direct physiological substrates of kinases so that anti-phosphosite antibodies can be derived to determine if exploratory kinase inhibitor drugs are truly hitting their intended targets in patients. A recent study demonstrated convincingly that the Snf-1-related serine/threonine kinase Hunk is essential for autochthonous tumor metastasis in a MYC-driven breast cancer model. Another recent study reported the development of a novel, efficient approach to identify true physiological kinase substrates from any tissue or cell source termed the Reverse In-gel Kinase Assay (RIKA). The goal of the work in this project was to bring these two advancements together to identify Hunk substrates, providing the first look downstream of the only kinase known to be required for breast cancer metastasis and to inform strategies to block Hunk activity in breast cancer.

BODY

Task 1. To develop a Hunk Reverse In-gel Kinase Assay to identify physiological substrates in breast cancer cells

To achieve the goals of this aim, it was first necessary to generate recombinant, enzymatically active Hunk kinase. Our first strategy was to clone the open reading frame of Hunk, predicted to encode a 90 kDa protein, into the bacterial expression vector pQE80L, and to induce expression in E. coli strain BL21. We have previously used this strategy successfully to produce nine other active serine/threonine kinases in addition to several tyrosine kinases. Induction with IPTG was performed under a variety of conditions of time and temperature, and the nickel-affinity purification of the His-tagged Hunk kinase was attempted. Despite repeated attempts, we were unable to produce and enrich full-length Hunk under any condition. The major product enriched by nickel affinity enrichment was a ~30 kDa species that could not represent full length Hunk (Figure 1). To ensure that the inability to produce Hunk in E coli was not a function of the pQE80 vector or the BL21 strain we cloned the Hunk open reading frame into pBAD33. Similar results were obtained upon induction of

DH10B cells transformed with this construct (data not shown). Based on these data, we concluded that is was not feasible to generate active full-length Hunk kinase in E. coli.

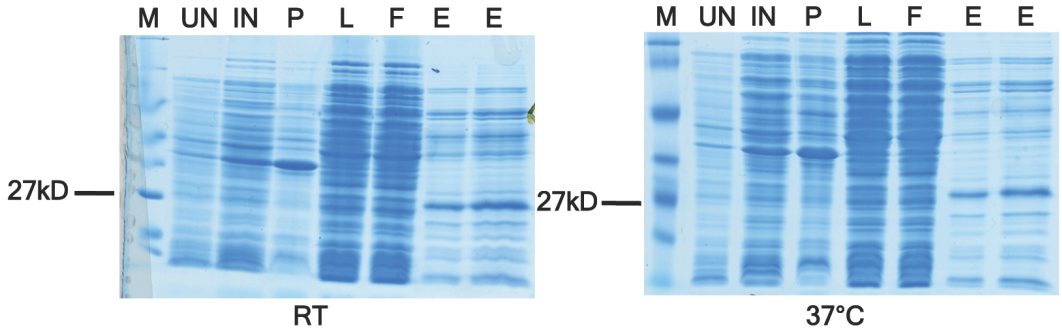

Figure 1. Expression and purification of Hunk protein from E. coli. To obtain Hunk protein for RIKA, the *Hunk* gene was cloned in the protein expression vector pQE80-L, and transformed into *E.coli* strain BL21. The expression of Hunk protein was induced with 0.4 mM of IPTG at either room temperature or at 37°C. The cells were harvested after 3 hours of induction. The cells were lysed using a French Press. The lysates were centrifuged at 20,000 X g for 30 minutes to separate into two fractions, the supernatant and the pellet. Hunk protein was purified with nickel affinity resin from the supernatant. The predicted molecular weight of Hunk protein is ~90 kDa. However, the protein enriched in the eluate was ~30 kDa, suggesting that the expressed Hunk protein was degraded *in E. coli* BL21. Hunk was then cloned into another expression vector (pBAD33), and transformed into *E. coli* DH10B. Hunk expression was induced with arabinose and purified with nickel affinity resin. A degradation product of similar molecular weight was obtained (data not shown). These results strongly suggest that Hunk cannot be expressed in *E. coli* as a full-length protein. **M**, molecular weight marker; **UN**, un-induced lysate; **IN**, induced lysate; **P**, pellet fraction; **L**, lysate loaded to the nickel resin column; **F**, lysate flow through; **E**, eluate fraction; **RT**, room temperature.

To overcome this technical impasse, we established the baculovirus insect cell expression system in the lab. The Hunk open reading frame was cloned into pFast-Bac1 and transfected into Sf9 cells. Large-scale cultures were infected with the Hunk-expressing baculovirus, and a ~90 kDa species was affinity purified using nickel resin (Figure 2). To determine if the Hunk kinase purified from insect cells was enzymatically active, an in vitro auto-phosphorylation assay was performed using radiolabeled ATP. Robust autophosphorylation of the ~90 kDa species was observed (Figure 3). We concluded that the insect-cell produced recombinant Hunk kinase was active and proceeded to develop a Hunk RIKA.

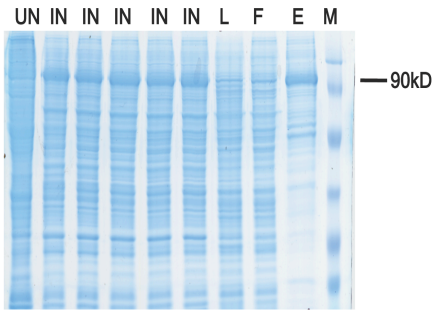

Figure 2. Expression and purification of Hunk protein from insect cells. To obtain full-length and active Hunk protein, the gene was cloned into the insect expression vector pFast-Bac1. Baculovirus containing the Hunk open reading frame was produced, and the expression of Hunk protein was achieved by infecting insect cells Sf9 with baculovirus. Hunk protein was purified using nickel affinity resin. As shown in **Figure 2**, a protein with an apparent molecular weight of approximately 90 kDa was enriched in the eluate fraction. **UN**, protein lysate from uninfected Sf9 cells; **IN**, protein lysate from Sf9 cells infected with baculovirus; Hunk expression efficiency under various infection conditions was tested. **L**, lysate loaded to the nickel affinity column. **E**, enriched Hunk protein eluted from the affinity column. **M**, protein molecular weight marker.

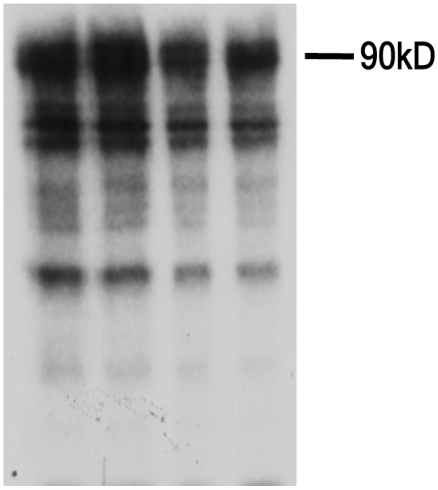

Figure 3. Hunk protein expressed in Sf9 cells is active. To test whether Hunk purified from Sf9 cells was active, an in vitro kinase assay was performed. Hunk protein was mixed with 20 μM cold ATP, 1 μCi γ-^{32}P-ATP in kinase reaction buffer containing 20 mM Tris, 20 mM $MgCl_2$. The mixture was incubated at room temperature for 2 hours. The reaction was stopped and analyzed by SDS-PAGE. The phosphorylated Hunk protein was transferred to PVDF membrane that was dried and exposed to X-ray film. As shown in Figure 3, a protein with a molecular weight of ~90 kDa was strongly phosphorylated.

To determine whether Hunk could regain its enzymatic activity after denaturing gel electrophoresis, we performed a RIKA under standard conditions using Hunk purified from both the soluble and insoluble fractions of infected Sf9 lysates. Hunk from both sources was able to auto-phosphorylate in the gel after refolding using a descending urea gradient (Figure 4). These data demonstrate that Hunk can regain its activity after denaturing PAGE.

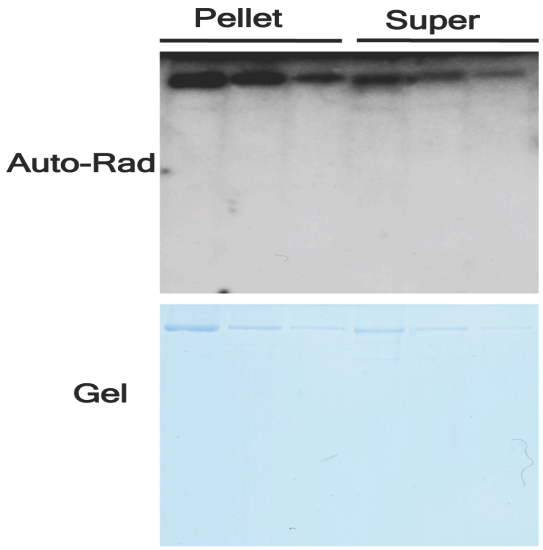

Figure 4 . Hunk can be refolded efficiently in-gel. Prior to profiling Hunk kinase substrates by RIKA, we tested whether Hunk could be efficiently refolded in-gel. Sf9 cells transfected with baculovirus were lysed. The lysates were separated into two fractions, the supernatant and the pellet, by centrifuging at 20,000 X g for 30 minutes. Hunk protein was purified from both the pellet and the supernatant under denaturing conditions. The purified Hunk was analyzed in an in-gel kinase assay. The gel was stained with Coomassie blue, dried and exposed to X-ray film. As shown in Figure 4, Hunk from both the pellet and the supernatant is strongly auto-phosphorylated. **Pellet**, Hunk purified from Sf9 cell lysate pellet; **Super**, Hunk purified from Sf9 cell lysate supernatant.

To determine if Hunk could detect a known substrate in a RIKA, a titration of Hunk was electrophoresed on a one-dimensional Hunk RIKA gel containing 50 ug/ml Hunk. As shown in Figure 5, Hunk was specifically detected in the RIKA.

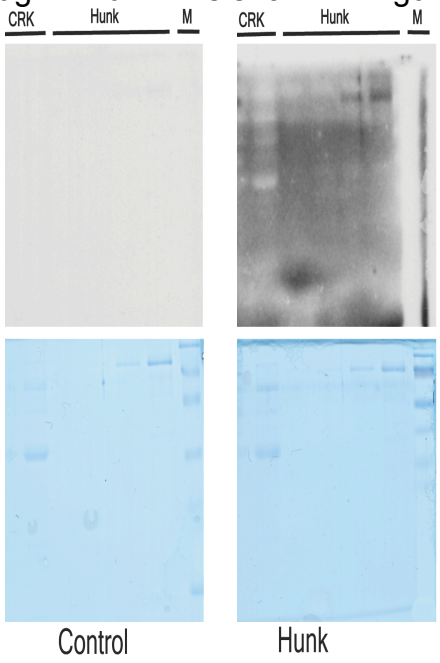

Figure 5. Developing a functional Hunk RIKA. To develop a Hunk RIKA, a gel containing 50 ug/ml Hunk was generated. Recombinant CRK and Hunk were used as negative and positive controls respectively. A parallel assay was performed using a gel containing no kinase Hunk was clearly detected in the assay. **M**, protein molecular weight marker.

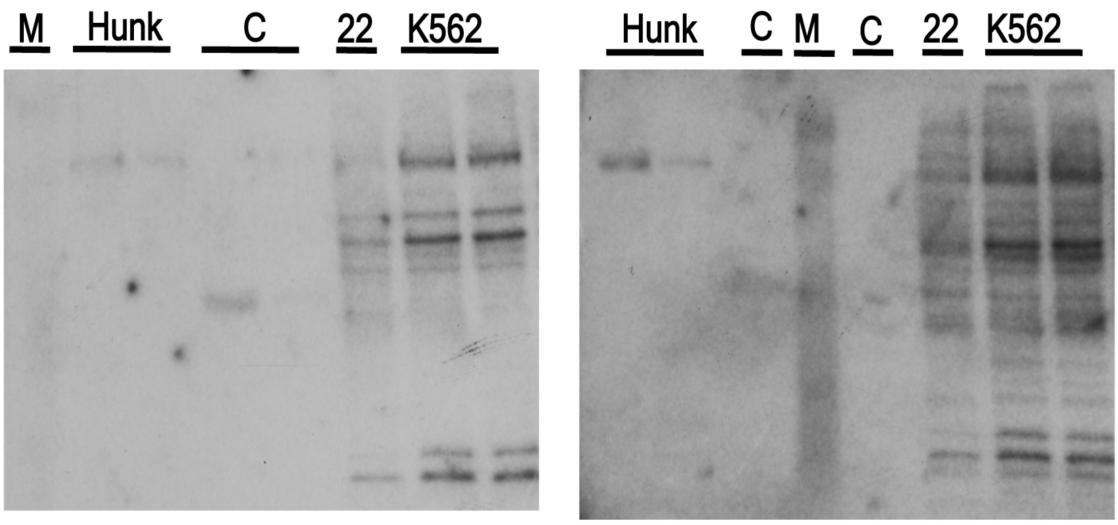

Figure 6. Profiling Hunk substrates using an on-membrane kinase assay. K562 lysates and 22RV1 lysates were resolved by SDS-PAGE. The proteins were transferred to a PVDF membrane. After the proteins on the membrane were refolded using a descending urea gradient, the membranes were incubated with γ-^{32}P-ATP in the presence (right gel) or absence (left gel) of recombinant Hunk in the kinase reaction buffer. In the presence of Hunk, multiple new species were observed. Recombinant Hunk and CRK were used as positive and negative controls respectively. **K562**, K562 cell lysate; **M**, protein molecular weight marker; **C,** CRK, **22**, 22RV1 cell lysate.

The difficulties we encountered in purifying sufficient quantities of active Hunk kinase to perform Hunk RIKAs prompted us to determine whether the assay could be performed in solution to detect substrates after transfer to a membrane. We worked out key parameters to allow us to specifically and sensitively detect Hunk substrates with a high signal-to-noise ratio in an on-membrane, as opposed to an in-gel, reverse kinase assay (Figure 6). This represents a significant step forward technically, allowing us to perform the assy using nearly one-hundred fold less Hunk in each assay.

To determine if the Hunk RIKA could detect Hunk substrates in the human proteome, whole cell extracts were analyzed in an on-membrane Hunk RIKA. A parallel membrane with no kinase reaction served as a negative control. Multiple bands were specifically phosphorylated in the Hunk-containing gel, representing potential Hunk substrates (Figure 5). Due to time constraints, this assay was peformed using cell extracts from K662 and 22RV1 cells that were on hand at the time of the experiment. We are currently extending these experiments to whole cell and fractionated extracts from breast cancer lines, including MCF7.

Task 2. To identify Hunk substrates that respond rapidly and robustly to Hunk knockdown by becoming hypo-phosphorylated

Experiments to identify Hunk substrates by mass spectrometry are currently in progress.

Task 3. To identify Hunk phosphoacceptor sites on true physiological substrates

Due to time constraints, this aim has not been achieved to date.

Task 4. To derive and test in cells competitive inhibitory peptides mimicking Hunk phosphoacceptor sites

Due to time constraints, this aim has not been achieved to date.

Task 5. Final analyses and report writing.

Completed.

KEY RESEARCH ACCOMPLISHMENTS

- Produced active, full-length Hunk kinase in insect cells
- Developed a Hunk-specific reverse in-gel kinase assay
- Developed a high sensitivity Hunk-specific on-membrane reverse kinase assay

REPORTABLE OUTCOMES

Bieberich, C.J. and Li, X. **Identifying physiological substrates of the Snf-1-related kinase Hunk.** Poster presentation**,** 6th Era of Hope Conference, August 2-5, 2011, in Orlando, Florida.

CONCLUSIONS

During the past year, we were successful in overcoming several daunting technical hurdles to produce active, full-length Hunk. We also succeeded in developing a functional Hunk reverse in-gel kinase assay to detect physiological substrates of this metastasis-associated kinase. We dramatically increased the sensitivity of the assay by developing an on-membrane approach that also requires far less active kinase to perform. Although the granting period has ended, we are continuing to pursue our goals to identify physiological Hunk substrates, and to develop peptide-based inhibitors to block Hunk activity in breast cancer cells. We remain fully committed to achieving these goals, and anticipate success in the coming few months.

REFERENCES

None

APPENDICES

None

BIBLIOGRAPHY

Bieberich, C.J. and Li, X. **Identifying physiological substrates of the Snf-1-related kinase Hunk.** Poster presentation**,** 6th Era of Hope Conference, August 2-5, 2011, in Orlando, Florida.

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